Study of gene polymorphism for stromal cell-derived factor 1 (SDF1/XCL12) and its elevation in plasma of women of reproductive age with pelvic inflammatory diseases in Babylon province

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Abstract

**Aims:** To discover possible relationships between CXCL12 single nucleotide polymorphisms (SNPs) and determine an elevation of SDF-1α in patients with PID.

**Methods:** Veins blood samples were aseptically collected from women of reproductive age with pelvic inflammatory disease (PID) and healthy control, attending in two hospitals of Babylon Province: Province: Babylon Maternity and Pediatrics Hospital, and Al-Mahaweel General Hospital, during the period from January to October 2018. The enzyme-linked immunosorbent assay and polymerase chain reaction–restriction fragment length polymorphism and bacterial culture identification were, respectively, used to measure the plasma stromal cell-derived factor 1α level, stromal cell-derived factor1 polymorphism in 45 healthy controls and in 43 patients with pelvic inflammatory disease before they received treatment protocols.

**Results:** The concentration of plasma stromal cell-derived factor 1α was higher in patients with pelvic inflammatory disease compared to normal controls. There were significant correlations between plasma stromal cell-derived factor 1α level and white blood cell count in addition to between stromal cell-derived factor 1α level and neutrophil count in females with pelvic inflammatory disease. There was no significant different distribution of stromal cell-derived factor 1 genotypes between women with pelvic inflammatory disease and normal healthy controls. Patients with pelvic inflammatory disease having stromal cell-derived factor 1-3’A allele were associated with significant different higher level of plasma stromal cell-derived factor 1α compared to patients with pelvic inflammatory disease having G/G homozygous alleles (P = 0.01). In healthy control, there was no significant difference in the level of plasma stromal cell-derived factor 1 concentration between women with and without stromal cell-derived factor 1-3’A allele. The concentration of CXCL12 was elevated in the patients with pelvic inflammatory disease than control healthy women and there were significantly different between them. In conclusion, A novel linkage and association was found between genetic variation of CXCL12 and pelvic inflammatory disease progression.

**Key words:** Stromal cell-derived factor 1α (SDF-1α), pelvic inflammatory disease (PID), single-nucleotide polymorphism (SNP), CXCL12

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Introduction

The term “pelvic inflammatory disease” refers to the fact that is an infection and inflammation of a woman's pelvic organs including the uterus (womb), Fallopian tubes (tubes), cervix, and ovaries. PID is very common and is evaluated to affect around 1 million females with reproductive age every year in the US (1).

Pelvic inflammatory disease usually develops as the result of spread of sexually-transmitted disease. Some women with PID may also have indication of lower genital tract infection too. The infection usually begins in the lower genital tract and ascends to the upper genital tract over time. Commonly, women with lower genital tract infections do not have any symptoms and so do not seek care. It is the asymptomatic nature of these infections that permits the microbial pathogens involved to finally enter the upper genital tract and cause infection there (2).

Several different bacteria may cause infection female genital tract infection, but the most common chlamydia and gonorrhea. Chlamydia and gonorrhea are the two most common bacterial sexually transmitted infections, which are caused by *Chlamydia Trachomatis* and *Neisseria gonorrhoeae* respectively; these pathogens are isolated from the superior genital tract of up to a half of women with PID (3).

Diagnostic criteria for PID are oral temperature >38.3°C, abnormal cervical mucopurulent discharge presence of abundant numbers of white blood cells, C-reactive protein level and leukocytosis (4).

Some remarkable differences among persons are observed in the clinical course of inflammations or infections. Chemokines are members of a superfamily of small proteins (7–14 kDa) of pro-inflammatory mediators with chemoattractant properties whose main documented role is leukocyte recruitment at inflammatory sites. They have been involved in different activities, including regulation of inflammation (5). Stromal cell derived factor (SDF) 1-alpha (also known as CXCL12) is chemokine that is implicated in immune cell activation, differentiation, and migration, wound healing, and tumorigenesis, CXCL12 is an essential chemokine during development and is critical for the homeostatic regulation of immune cells trafficking and tissue regeneration (6,7). CXCL12 as a ligand interacts with chemokine (C-X-C motif) receptor 4 (CXCR4) and transduce signals implicated in essential cellular processes such as differentiation, apoptosis and leukocytes chemotaxis, the chemokine (C-X-C motif) ligand 12 (CXCL12) is selectively expressed on B and T lymphocyte and is located on chromosome 10q11.1 (8).

Single nucleotide polymorphism (SNP) rs1801157 also recognized as G801A is located on exon 4 of β splice variant in CXCL12 gene transcripts. This SNP involves a guanine to adenine (G → A) substitution at base pair 801 of the 3'-untranslated region of CXCL12 gene which is associated with affect levels of CXCL12 production *in vitro* and *in vivo* progression in various studies (9). The allelic and genotypic frequencies of SNP rs1801157 of CXCL12 gene are studied to increase the interaction with CXCR4. This known interaction has been exposed to enhance pathogenesis and disease susceptibility of multiple diseases (10).

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Finally, Pelvic inflammatory disease is caused by microbial pathogens that colonize in the endocervix and move to the endometrium and fallopian tubes. Neutrophils and T-lymphocytes are plentiful in the PID patients inflammatory lesions and a neutrocytosis is found in patient’s blood (11,12). Many upper genital tract infections, such as endometritis, pelvic peritonitis, tubal abscess, and salpingitis, are so presented (13). Thus, we hypothesized that the genotypic frequencies of SDF-1-3’A induce expression of SDF-1 are associated with PID. No study had been conducted on the correlation between plasma concentration of SDF-1a protein and PID, in addition to be compared the impact of the SNP of SDF-1 concentration in PID Iraqi patients, we measure the plasma levels of the SDF-1 protein in patients with PID and in healthy control participants. Therefore, we related SNP of SDF-1 with PID, then demonstrated whether the SDF-1α expression levels were changed among SDF-1 different polymorphism alleles and pathogenicity of bacteria in PID patients.

Materials and methods

Subjects of the Study and specimen collection:

This research was a based case-control study Clinical samples were collected from patients admitted to the consultant clinics of Gynecology and Obstetrics, in two hospitals of Babylon Province: Babylon Maternity and Pediatrics Hospital, and Al-Mahaweel General Hospital, during the period from January to October 2018. This study enrolled 88 women (43 PID patients and 45 healthy controls) were subjected for sampling which include both endocervical swab and venous blood specimen from each female. The age of women ranged from 18 to 50 years. 43 patients who were diagnosed to have PID by the gynecologist as having PID, according to the characteristic criteria of national guidelines for pelvic inflammatory disease (14, 15) and according to the signs and symptoms, pelvic and abdominal ultrasound. Except for PID, 50 healthy women, the controls, were matched to 44 patients with PID in sociodemographic and clinical data such as occupation, socioeconomic, area, alcohol drinking, cigarette smoking, and age. There was no significant difference ($P= 0.457$) in age distribution between control women and patients with PID (Table 1). Any predisposing factor, which was associated with PID, was excluded in the selection of control. In this study, females with recent usage (with in 7 days) of antibiotic treatment; pregnant females; malignancy; severe neutropenia (WBC count lower than $1.0 \times 10^9$ cells/L); organ or bone marrow transplant; HIV infection. Moreover, intake of anti-inflammatory drugs like corticosteroids was also excluded from sampling.

The blood samples were obtained from all Participants by a sterile disposable syringe and dispersed into the sterile EDTA-tubes were utilized for determining blood parameters such as, white blood cell (WBC) and neutrophil, C-reactive protein (CRP) (16) and SDF-1α expression, in addition to be used for processing of human DNA purification and genetic studies, the blood samples obtained for measurement of SDF-1α were undergone centrifugation where the plasma were obtained and stored at -20°C with genomic DNA extraction samples until it be used.

The study was performed with the approval of the Committee in College of Medicine/ Babylon university, Babylon Maternity and Pediatrics Hospital, Al-Mahaweel General Hospital, and informed written agreement was obtained from each participants.

Measurement of the Plasma SDF-1 Level by Using Enzyme-Linked Immunosorbent Assay. An enzyme-linked immunosorbent assay (ELISA) was used to measure the plasma concentrations of SDF-1 in all blood plasma samples (Elabscience Biotechnology Co, China). Each plasma sample (100 μL) was directly transferred to the microplate strip well of the ELISA plate and then incubated for 90 min at 37°C. The liquid was removed, the detection antibody was added, and the reaction system was incubated for 60 min at 37°C. Antibody binding was detected using streptavidin-streptavidin.
conjugated horseradish peroxidase (HPR) and developed using a substrate solution for 15 min at 37°C. The reaction was then stopped, and the optical density (OD) was determined using a microplate reader set at 450nm. Soluble SDF-1 concentrations were quantitated according to a calibration curve using a human SDF-1 standard.

**Genomic DNA Extraction from Frozen Blood Sample:** Frozen blood samples were stored at -20°C and taken from them should be thawed in water bath at 37°C for 15 minutes before it be used. DNA was extracted as per (Geneaid, UK) protocol according to the manufacturer’s instructions. DNA was dissolved in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA•Na2) and then quantitated by Nano Drop spectrophotometer at OD260. Final preparation greater than 1.8 was stored at -20°C and used as templates for polymerase chain reaction (PCR).

**Polymerase Chain Reaction _restriction Fragment Length Polymorphism:** The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to genotype the G801A polymorphism in the SDF-1α gene. The sequences of primers used to amplify the SDF-1α G801A genotype were 5'-CAGTCAACCTGGGCAAAGCC-3' (forward) and 5'-CCTGAGAGTCCTTTTGCGGG-3' (reverse; GenBank accession number L36033). PCR was performed in a 5µL volume DNA template, (2.5 µl) from each (Macrogen, Korea) forward and reverse primer then (12.5 µl) from (Promega, USA) master mix were added to each microcentrifuge tube. The reaction mixture was adjusted to (25 µl) using (Bioneer, Korea) nuclease free deionized distilled water and then put in shaker and spinner for 10 cycles for better mixing. After mixing, the mastermix tubes were transferred to the thermocycler (Clever Scientific LTD/UK) which is previously programmed with the above protocol according to the gene to be amplified, the PCR cycling conditions were 5 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C, with a final step at 72°C for 20 minutes to allow a complete extension of all PCR fragments. 15µl from SDF-1α G801A PCR products was mixed separately with a 5µl 1X NEB buffer (New England, Biolabs), and 5µl of Hpa II (5U) (New England, Biolabs). The reaction was adjusted to 40µl using sterile deionized H2O. The solution was mixed by flicking, followed by spinning in microcentrifuge at 5000 rpm for 30 sec, then incubated at 37°C for 5 hours. After digestion, DNA fragments were electrophoresed on a 3% agarose gel containing ethidium bromide (0.5 µg/ml). The bands were then visualized by photographed using digital camera. The amplified products were determined by comparison with a 100 bp ladder (Promega, USA). As a result, SDF-1 wild-type alleles yielded 100-bp and 193-bp products, while SDF-1 A alleles yielded a 293-bp product.

**Statistical Analysis:**
Experimental results are showed as the mean±SE. Hardy-Weinberg equilibrium was assessed using a goodness-of-fit $\chi^2$ test for biallelic markers. A Mann-Whitney U test was used to compare the differences in plasma SDF-1α level, WBC, and neutrophil counts as well as CRP level between the healthy women and patients with PID. Spearman’s rank correlation analysis was used to estimate the correlations between plasma SDF-1α level and inflammatory markers WBC count and neutrophil count in PID patients. Fisher exact test was used to test the difference in genotype frequencies of SDF-1 gene polymorphism between patients with PID and normal controls. A Kruskal-Wallis test was used to detect the difference in concentrations of plasma SDF-1α protein among 3 genotypes of SDF-1α gene polymorphism. The data were analyzed by using version 23 SPSS statistical software.

**Results and discussion:**
The analysis in this study was based on a sample of 88 women. Except for PID, 45 healthy women, the controls, were matched to 43 patients with PID in demographic and clinical laboratory data such as, socioeconomic, occupation, cigarette smoking, alcohol drinking. Age and nonspecific inflammatory markers such as white blood cells (WBCs), neutrophil counts, and C-reactive protein, all participants were examined the differences by mean and standard error.
Health descriptive statistical test. In the meantime, there was no significant difference ($P=0.457$) in mean of age distribution between control women ($28.2 \pm 0.87$ years) compared with PID patients ($29.9 \pm 1.14$ years). The WBC, neutrophil count, and CRP level were significantly elevated in patients with PID ($10718.8 \pm 719.08$ Cells/mm$^3$), ($7987.1 \pm 538.5$ Cells/mm$^3$), and ($58.4 \pm 4.7$ mg/L) respectively before they received treatment compared with those in normal controls ($5451.1 \pm 187.5$ Cells/mm$^3$), ($2927.9 \pm 112.8$ Cells/mm$^3$), and ($4.8 \pm 0.4$ mg/L) respectively ($P<0.001$), as listed in Table (1).

Table (1): The Demographical and laboratory data of both controls and patients with PID $^a$

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>PID (n = 43)</th>
<th>Control (n = 45)</th>
<th>$P$ Value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td></td>
</tr>
<tr>
<td>Occupation</td>
<td>None, housewife</td>
<td>None, housewife</td>
<td></td>
</tr>
<tr>
<td>Socioeconomic</td>
<td>Middle</td>
<td>Middle</td>
<td></td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Alcohol drinking</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td>$29.9 \pm 1.14$</td>
<td>$28.2 \pm 0.87$</td>
<td>0.457</td>
</tr>
<tr>
<td>WBC (/mm$^3$)</td>
<td>$10718.8 \pm 719.08$</td>
<td>$5451.1 \pm 187.5$</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Neutrophils (/mm$^3$)</td>
<td>$7987.1 \pm 538.5$</td>
<td>$2927.9 \pm 112.8$</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>$58.4 \pm 4.7$</td>
<td>$4.8 \pm 0.4$</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

$^a$The statistical difference was analyzed by the Mann-Whitney $U$ test.$^bP<0.05$ was considered significant.

These results showed that the concentrations of plasma SDF-1α in healthy controls as well as in patients with PID before they received treatment protocols are shown in Figure (1). Plasma SDF-1α level in pretreatment patients with PID ($2829.3 \pm 182.8$ pg/ml) was significantly greater ($P<.0001$) than that of normal controls ($1848.7 \pm 85.9$ pg/ml).

Figure (1): Plasma stromal cell-derived factor 1α (SDF-1α) concentrations were analyzed in pretreatment plasma of 43 patients with pelvic inflammatory disease (PID) and 45 controls. The significantly different ($P<.0001$) expression of plasma SDF-1 was shown between 45.

Moreover, the correlations between plasma SDF-1α level and WBC count as well as between SDF-1α level and neutrophil count in patients with PID before they received treatment are shown in Figure (2). A significant correlation
between plasma SDF-1α level and WBC count (Spearman correlation coefficients $r=0.588$, $P<0.001$, $n=43$) as well as a significant correlation between SDF-1α level and neutrophil count ($r = 0.565$, $P < .001$, $n=43$) were found.

Figure 2: The correlations among plasma stromal cell-derived factor 1a (SDF-1a) concentration, white blood cells (WBCs), and neutrophil count in 43 patients with pelvic inflammatory disease (PID) before they received the treatment protocols. There is a significant correlation between plasma SDF-1a level and WBC counts (Spearman correlation coefficients $r=0.588$, $P<0.001$) as well as a significant correlation between SDF-1a concentration and neutrophil count ($r=0.565$, $P < .001$) in 43 patients with PID before they received the treatment protocols.

It has been shown that SDF-1α is one of the chemokines responsible for attracting and accumulating T-lymphocytes (17), as well as responsible for the activation, adhesion, and migration of neutrophil leukocytes to inflammatory sites (18). Elevated production of SDF-1 has been shown to be associated with some of the infectious and inflammatory diseases (19). (20) Found that an elevated plasma SDF-1 concentration can be used as a biological marker for the early diagnosis of Community-Acquired Pneumonia and for the early detection of its severity. (21) Reported that SDF-1 expression was significant association with distant metastasis in breast cancer and majority of SDF was produced by cancer cells. (22) Also showed that SDF-1 was considered a crucial cytokine causing osteoarthritis disease and was likely associated with the progression of inflammation. In this study, revealed that plasma SDF-1α concentration was significantly elevated in patients with PID before they received treatment compared to healthy women as a control.

Pelvic inflammatory disease is a importance of either a sexually transmitted infection or medical procedures that destroy the barrier of the cervix (23). For the duration of the pathogenesis of PID, neutrophils and T-lymphocytes migrate to inflammatory sites for acting as a defense against pathogens (24). Moreover, the concentration of neutrophils is elevated in patients with PID (25), and that T-lymphocytes produce proinflammatory cytokine, type 1T helper cell (Th1) cytokines, or Type 2 T helper cell (Th2) cytokines has been reported to be related to PID (26,27). We hypothesized that the elevated plasma CXCL12-α is responsible for the pathogenesis of PID. Stromal cell-derived factor -1α activates and recruits neutrophils to inflammatory sites for defense against pathogens (28), as well, the activated T-lymphocytes consequently induce proinflammatory cytokines (29), Th1 cytokines, and Th2 cytokines production for an immune response in PID (30,31). This observation suggests that CXCL12 – α expression is implicated in the pathogenic process of PID.
The PCR-RFLP products and genotype distributions of SDF-1α are shown in Figure (3) and in Table 4, respectively. In this study population was in Hardy-Weinberg equilibrium ($P > 0.05$, $\chi^2 = 21.13$). There was no significantly different distribution of SDF-1 genotypes between patients with PID and healthy women Table (4), $P = 0.544$

![Figure 3](image-url)

**Figure (3):** 2% Agarose gel electrophoresis at 70 volt for 50min, showing the Polymorphism analysis of the $SDF-1\alpha$ gene by polymerase chain reaction (PCR) followed by specific restriction enzyme digestion patterns of $SDF$ - 801G/A polymorphisms of cXcL12 gene using Hpa enzyme. L: 1500 DNA ladder. Lanes 1, 3, 7, 12, 15, 16, 17 : homozygous wild type (GG). Lane 2, 4, 5, 8, 10, 11, 13, 14 : heterozygous genotype (GA). Lanes : 6, 9 homozygous mutant genotype (AA).

**Table (4):** The Genotype Frequencies of Stromal Cell-derived Factor 1 in Patients With PID and Controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients n (%)</th>
<th>Controls n (%)</th>
<th>$P$ Value $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>28 (65.1)</td>
<td>24 (53.3)</td>
<td></td>
</tr>
<tr>
<td>G/A</td>
<td>11 (25.6)</td>
<td>16 (35.6)</td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>4 (9.3)</td>
<td>5 (11.1)</td>
<td>0.544</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The statistical significance was analyzed by Fisher exact test. $^bP$ value <.05 was considered significant at 95% C.I.

As well, there was no significant difference in plasma SDF-1α concentrations among the 3 genotypes (Table 5) in both healthy women ($P = 0.313$) and patients with PID ($P = 0.957$). Also, categorized the individuals with at least one mutated A allele as one subgroup and observed the individuals with homozygous G/G alleles as another subgroup and found that plasma SDF-1α concentration was significantly elevated in patients with PID having A/A or A/G alleles of SDF-1 gene compared with those with G/G alleles ($P = 0.01$; Table 5). But this finding was not demonstrated in normal controls ($P = 0.124$).

**Table (5):** The Plasma Concentrations (pg/mL) of Stromal Cell-derived Factor 1α in Different Genotypic Groups.

<table>
<thead>
<tr>
<th>Genotypic Frequencies</th>
<th>Patients With PID (n = 43)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%) mean ± SE</td>
<td>n (%) mean ± SE</td>
<td></td>
</tr>
</tbody>
</table>

Three different genotypes
It has been shown that SDF-1-3′A allele of SDF-1 gene is associated with the risk of diseases or acceleration of disease progression (32,33,34) in addition to be associated with alteration of plasma SDF-1 protein expression (35,36). Results of this study, found that there were no significantly different genotype distributions of SDF-1 between patients with PID and healthy controls. In contrast, showed a significant elevation of plasma SDF-1α concentration in the patients with PID having A/A or A/G alleles of SDF -1 gene compared with patients with PID having G/G alleles. (37) reported that SDF-1 isoforms only SDF-1β mRNA was significantly over expressed 2.5-fold to six fold in bladder cancer compared with expression of SDF-1α in the bladder tissues. (38) reported that these genotypes showed no significant difference in the levels of the SDF-1α and β mRNA,so suggested that SDF-1α and SDF-1β may act as tumor markers as well as no alteration of the ratio of SDF-1α and β mRNA. However, (39) and (40), demonstrated no clear association between SDF-1α and β mRNA expression and SDF-1-3′ A genotype in normal donors, but significantly excessive SDF-1 expression in HIV-1 infected children with SDF-1-3′ A polymorphism compared to those with SDF-1-wild-type. Their finding was comparable to this result that SDF-1-3′ A polymorphism was associated with the elevated CXCL12-α expression in patients with PID. It has been suggested that post-transcriptional regulation of the 3′ untranslated region (3′ UTR) is a powerful regulatory process that determines the rate of protein translation from messenger RNA. Regulatory elements targeting the 3′ UTR include microRNAs, single nucleotide change in the 3′ UTR can result in dysregulated post-transcriptional regulation, these SNPs disrupt the interaction sites for miRNA binding, which usually leads to stabilization of the mRNA transcript and increased SDF1 protein levels synergistic interaction with some microbial virulence factors,such as pathogens toxin, may cooperate with SDF-1-3′ A allele to induce production of plasma SDF-1α protein in patients with PID by increasing the half-life of the transcript but these factors may lack or are not implicated in the production of SDF-1α protein in the presence of SDF-1-3′ A allele in normal control.

**Conclusion:** Pelvic inflammatory disease is a major health problem in developing countries of the world,elevated plasma SDF-1a expression was involved in the pathogenic process of PID, genetic polymorphisms and virulence factors of genital tract pathogens play important role for SDF1-α expression and a risk for PID progression.
Reference: